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REVIEW

An expandable donor-free supply of functional hepatocytes for Toxicology

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The B-13 cell is a readily expandable rat pancreatic acinar-like cell that differentiates on simple plastic culture substrata into replicatively-senescent hepatocyte-like (B-13/H) cells in response to glucocorticoid exposure. B-13/H cells express a variety of liver-enriched and liver-specific genes, many at levels similar to hepatocytes in vivo. Furthermore, the B-13/H phenotype is maintained for at least several weeks in vitro, in contrast to normal hepatocytes which rapidly de-differentiate under the same simple - or even under more complex - culture conditions. The origin of the B-13 cell line and the current state of knowledge regarding differentiation to B-13/H cells are presented, followed by a review of recent advances in the use of B-13/H cells in a variety of toxicity endpoints. B-13 cells therefore offer Toxicologists a cost-effective and easy to use system to study a range of toxicologically-related questions. Dissecting the mechanism(s) regulating the formation of B-13/H cell may also increase the likelihood of engineering a human equivalent, providing Toxicologists with an expandable donor-free supply of functional rat and human hepatocytes, invaluable additions to the tool kit of in vitro toxicity tests.

Origin of the B-13 cell line

The AR42J-B13 (henceforth referred to as the B-13) cell line was sub-cloned from the rat adenocarcinoma AR42J cell line in the late 1970s. The AR42J cell line is an exocrine pancreatic adenocarcinoma cell line still used to the present day in basic exocrine pancreas research.¹⁻⁶ The AR42J cell line was derived from studies in which inbred Wistar/Lewis rats were treated with azaserine (O-diazoacetyl-L-serine).⁷ Azaserine is a naturally occurring antibiotic first isolated from *Streptomyces fragilis*⁸ that causes cytotoxicity in cells through at least 2 mechanisms:- inhibition of N-formylglycineamidine ribotide synthetase (PFAS, phosphoribosylformylglycinamidine synthase), which results in an inhibition of purine biosynthesis;⁹ and through spontaneous decomposition to yield diazoacetate, which carboxymethylates DNA primarily on the O₆ and N₇ of guanine and the N₃ of adenine.¹⁰⁻¹² Azaserine is also an inhibitor of glucosamine-6-phosphate isomerase (GFPT1)⁹ and is often used experimentally in studies on hexosamine biosynthesis.¹³ A single intraperitoneal injection of 30 mg azaserine/kg body weight is capable of inducing adenoma and subsequently carcinoma in rats several months after exposure, primarily in the pancreas.¹⁴ Accordingly, azaserine has been

used in the past to generate experimental models of pancreatic cancer.¹⁵⁻¹⁹ Why the pancreas is a target organ for tumours with azaserine however, remains unclear.

AR42J cells were isolated from tumour cells generated from the rat azaserine studies of Longnecker et al⁷ and were transplanted back into untreated rats of the same strain by subcutaneous and intraperitoneal injections up to five times. The transplanted cells were reported to be capable of forming tumours at the site of injection and in lung and liver in some recipients.⁷ The AR42J cell line generated was noted to express high levels of amylase and other pancreatic exocrine enzymes.²⁰ For summary, see Fig. 1.

The B-13 cell line was sub-cloned from the AR42J cell line by the Kojima lab in 1996 and was reported to differentiate into insulin-producing pancreatic endocrine β -like cells in vitro after treatment with hepatocyte growth factor (HGF) and activin A.²¹ More recently, this effect has been shown to require expression in B-13 cells of transcription factors Ngn3 together with Nkx6.1 or MafA.²²

In 2000, Shen et al observed that the B-13 cell underwent a profound alteration in phenotype from the pancreatic exocrine-like cell to an hepatocyte-like cell in response to glucocorticoid exposure.²³ In this study, a coordinated change in B-13

phenotype to an hepatocyte-like (B-13/H) cell - and not simply an occasional aberrant expression of an hepatocyte gene - appeared to be the case (see also Tables 1-3). A series of reports subsequent to this discovery essentially confirm these observations and demonstrate that B-13/H cells express a variety of genes that are hepatocyte-specific or hepatocyte-enriched, including expression of blood proteins, cytoskeletal proteins, metabolic enzymes, xenobiotic metabolising enzymes (both phase I and phase II) and transporter proteins (for details see Table 1-3).

Mechanisms regulating B-13 differentiation into hepatocyte-like cells

Progenitor nature of B-13 cells

The B-13 cell line readily proliferates in simple cell culture media such as Dulbecco's modified Eagle medium (DMEM) supplemented with serum (typically 10% (v/v)), with a doubling time of 30 \pm 7.3 hours.⁴⁶ The cells do not require any of the culture modifications often associated with stem cell maintenance (such as fibroblast feeders, matrigel or colony selection during sub-culture to select for progenitor characteristics). The cells will readily expand on plastic substrata and appear to have an altered - but stable - near tetraploid karyotype of male origin.²⁷ It is unknown for how long this karyotype has been stable, however B-13 cells have been repeatedly trans-differentiated into B-13/H cells for last 15 years, suggesting alterations at the genomic DNA level are very stable. B-13 cells do not grow in agar in vitro, and do not readily form tumours in SCID mice. However, B-13 cells are capable of selectively engrafting into the pancreas and liver after intravenous injection, and spontaneously forming hepatocyte-like cells in the liver.²⁷ B-13 cells therefore appear to retain some of the pathways that restrain dysregulated cell growth and differentiation. Were it not for their unique ability to differentiate into hepatocyte-like cells, B-13 cells might be considered no different from any other cell line.

The liver and pancreas are thought to contain progenitor cells localised in the ductal regions of the tissue (Fig. 2). Using the neural marker synaptophysin - which is expressed by B-13 cells and suppressed in B-13/H cells,²⁶ it is possible to identify putative progenitors in a limited number of both rat liver and pancreas ductal regions. The study of these progenitor cells in vitro is challenging since, in contrast to B-13 cells, the progenitor cells are difficult to purify and maintain as a pure population of progenitors. In the rat, liver progenitors (oval cells) are bi-potential and give rise to hepatocytes and biliary epithelial cells (cholangiocytes) in vivo.^{85,86} In our hands, rat oval cells in culture rapidly generate biliary epithelial cells and/or fibroblasts (possibly through epithelial-mesenchymal trans-differentiation of biliary epithelial cells) and form a minority of the cell population within a day or two of isolation (Fig. 3).

The data to date suggest that the B-13 may be equivalent to a pancreatic or liver progenitor cell since it trans-differentiates

from the B-13 acinar phenotype directly to the hepatocyte-like B-13/H phenotype. Using a B-13 cell line stably transfected with a green fluorescent protein (GFP) gene under control of a promoter (which is trans-activated in exocrine pancreas cells) Shen et al²³ showed that after exposure to glucocorticoid, cells could be identified that expressed both GFP and hepatocyte markers, suggesting that the mechanism of altered differentiation is likely a direct conversion from its "differentiated" pancreatic exocrine cell type directly to an hepatocyte, without reverting to a plastic progenitor-like intermediate). However, more recently, limited evidence suggests that B-13 cells may be directed to form a ductal (i.e. biliary-like) phenotype in response to glucocorticoid and EGF.⁸⁷ However, the identity of these cells is ambiguous, likely because the response lacks the quantitative trans-differentiation response seen with B-13/H cells and hepatocytes. This apparent plastic nature of B-13 cells may be reflective of the type of responses seen in a variety of cells in vitro, such as epithelial-mesenchymal transition (EMT), which commonly occur in vitro.^{88,89} However, whether B-13 cells enter (or are capable under certain circumstances of entering) a transient plastic state prior to conversion to B-13/H cells remains uncertain.

Mechanism(s) mediating the formation of B-13/H cells

There is good evidence to suggest that the glucocorticoid receptor (GR) mediates the response to glucocorticoid in B-13 cells and initiates the process of trans-differentiation. The synthetic glucocorticoid dexamethasone is frequently employed in generating B-13/H cells because it is less rapidly metabolised than natural glucocorticoids and is more selective for the GR than many natural glucocorticoids.^{29,30} However, B-13 cells respond similarly to a range other glucocorticoids and the response is inhibited by GR antagonists.^{23,90} Other classes of steroids (e.g. oestrogens) fail to promote the trans-differentiation of B-13 cells into B-13/H cells⁹⁰ (see also Fig. 4) and although glucocorticoids also activate the mineralocorticoid receptor,⁹¹ B-13 trans-differentiation to B-13/H cell does not occur in response to mineralocorticoids, nor is glucocorticoid-dependent trans-differentiation blocked by mineralocorticoid receptor antagonists.⁹²

Previous studies over the years have reported the appearance of hepatocyte-like tissue in pancreatic exocrine tissue in vivo, in response to a variety of pathological conditions (Table 4). More recent observations have focussed on the effects of glucocorticoid on pancreatic exocrine tissue differentiation. These studies have shown that both rodent and human embryonic acinar cells can be converted into hepatocyte-like cells in response to glucocorticoid in vitro.^{109,110} However, of fundamental importance from a practical point of view, the quantitative functionality of these pancreatic hepatocyte-like cells to hepatocytes from the liver was rarely made. When such comparisons were made, on a quantitative basis, it is arguable whether such cells could be defined as hepatocytes. However, this lack of quantitative comparability

may be a feature of the *in vitro* environment in which the cells were cultured, and does not indicate necessarily that the cells were not capable of forming functional hepatocytes in the correct environment (such as *in vivo*). This is supported by recent studies in both rodents and man.^{24,90}

Using *in vivo* models, Wallace et al demonstrated that treatment with high doses of glucocorticoid to adult rats for just a few weeks is enough to convert occasional pancreatic acinar cells into cells that express liver-levels of CYP2E1.²⁴ In a transgenic mouse with high circulating endogenous circulating glucocorticoids and which showed many of the clinical features of Cushing's disease, significant liver-levels of hepatocyte gene expression could be observed in the pancreata of many mice by adulthood (21 weeks of age).¹⁰⁷ The normal pancreas is therefore capable of altering to an hepatocyte-like phenotype that shows comparable levels of hepatocyte gene expression *in vivo* when exposed to pathologically high levels of glucocorticoid. Interestingly, these data also show that such a phenomenon is not restricted to embryonic or foetal tissue. Recent investigations also indicate that pancreata from adult patients maintained on high levels of systemic glucocorticoid therapy also express hepatocyte levels of hepatocyte expressed genes,⁹⁰ suggesting that the observations in rodents are likely translatable to man.

Although it was clear from the first observations by Shen et al that the liver-enriched transcription factor CCAAT/enhancer-binding protein- β (CEBP- β) was a pivotal downstream mediator of B-13 trans-differentiation to B-13/H cells,²³ the signalling pathways that lead from glucocorticoid to the induction of this transcription factor in B-13 cells are only recently being determined. Wallace et al provide strong evidence to suggest that GR activation in B-13 cells leads to a robust induction of the serine/threonine kinase SGK1⁹² and that this kinase, at least in part, phosphorylates the Wnt signalling protein messenger β -catenin as a component of the mechanism that precedes induction of transcription factors such as CEBP- β (see also Fig. 5c for schematic diagram of proposed proximal mechanism of trans-differentiation).^{48,29}

The physiological function of SGK1 *in vivo* is in the regulation of sodium salt re-absorption by the kidney. The kinase functions to mediate mineralocorticoid stimulation of renal sodium channel (ENaC) activity and anti-natriuresis through its phosphorylation of the ubiquitin ligase Nedd4-2, which leads to a block in epithelial ENaC ubiquitylation, a reduction in ENaC endocytosis and increased renal tubular epithelial sodium transport into the cell.¹¹¹ SGK1 mediates a stress response to low salt levels, promoting salt re-absorption. Accordingly, knockout of the *Sgk1* gene in mice has no overt effect on mice unless the mice are forced onto a low salt intake.^{112,113} To date, a role for SGK1 in cell differentiation and/or tissue development – with the exception of B-13 cells – has not been identified. However, a limited microarray study that compared the levels of approximately 5000 transcripts in B-13 and B-13/H cells identified SGK1 as the highest induced transcript in response to glucocorticoid treatment [supplementary data to Wallace et al²⁴]. Subsequent studies

demonstrated that expression of either of 2 human SGK1 variants (but not the “wild type” variant A) alone, drove B-13 trans-differentiation into B-13/H cells (see also Fig. 5).⁹² The SGK1 variants are identical with the exception of alternative N-terminal domains. These differentiation-promoting SGK1 variants are also induced in both mouse and human pancreatic acinar hepatic tissue or cells after exposure to high glucocorticoid.^{107,90} Using recombinant SGK1 and β -catenin, it was shown that SGK1 was able to phosphorylate β -catenin on the residues that target β -catenin for ubiquitination and degradation,⁹² a process that would be expected to down-regulate the Wnt signalling pathway. A Wnt signalling-responsive promoter-reporter gene construct suggested that Wnt signalling activity was highly active in B-13 cells.⁴⁸ Inhibition of Wnt signalling (using the inhibitor quercetin or through over-expression of a mutant β -catenin [pt-X β -cat]) or siRNA to knockdown β -catenin expression) alone resulted in Cebp- β induction and trans-differentiation of B-13 cells into B-13/H cells.⁴⁸ It is likely therefore, that specific *Sgk1* variants provide the link between GR activation and the Wnt signalling pathway, and that this crosstalk lies upstream of Cebp- β induction and trans-differentiation. In normal cells, this cross talk is unlikely to be operational and is only relevant in pathological situations such as chronic hyper-elevated glucocorticoid levels. However, the *Sgk1* cross talk with Wnt signalling may be amplified in some way in B-13 cells (e.g. through an over-induction and/or sustained elevation in SGK1 expression and/or a constitutively active PI3 kinase signalling pathway) such that the crosstalk mechanism is responsive in B-13 cells at physiologically normal concentrations of glucocorticoid.

Notable features of B-13/H cell formation

There are several features of the trans-differentiation of B-13 cells into B-13/H cells that are striking, particularly in the context of current attempts to generate functional hepatocytes from embryonic stem cells or induced pluripotent stem cells for use in hepatology or toxicology research.

Treatment with glucocorticoid is sufficient to promote the trans-differentiation of B-13 cells into B-13/H cells. This contrasts markedly with the range of different growth factors and other poorly defined additions (e.g. matrigel, fibroblast feeder layers) that are required or often used when ESCs or iPSCs are directed to differentiate into hepatocyte-like cells *in vitro*. Recombinant growth factors are also relatively expensive to produce and purify and are subject to variable functional activity, whereas glucocorticoids are inexpensive and relatively simple, stable chemicals.

A major physiological function of glucocorticoids is as a regulator of general metabolism. Glucocorticoids are synthesised by the adrenals and the levels secreted into the blood are controlled by the hypothalamus-pituitary-adrenal (HPA) axis.¹¹⁴ Glucocorticoids are stress hormones and high levels promote gluconeogenesis, lipolysis and protein and amino acid degradation.¹¹⁴ Glucocorticoids also promote

diuresis and have a general immunosuppressive (most notably, anti-inflammatory) effect, a feature that is often employed for therapeutic reasons.¹¹⁵ However, glucocorticoids also have a developmental role, at least within the foetus. Circulating levels of glucocorticoid are low in the embryo and foetus (placental enzymes such as 11 β -hydroxysteroid dehydrogenase [HSD2] metabolise glucocorticoids to inactive products and foetal levels only rise as the foetal HPA axis develops).^{116,140} This rise in circulating foetal glucocorticoid probably plays a major role in cell differentiation and tissue maturation, most notably in the lung, since premature babies are successfully treated with glucocorticoids to promote lung maturation.¹¹⁷

A biologically relevant GR activating concentration of glucocorticoids (typically 10⁻⁸ M) is sufficient to promote the trans-differentiation of B-13 cells into B-13/H cells. The affinity of the rat GR for dexamethasone is in the 10⁻⁹ M range (K_D = 2.3nM¹¹⁸) and typically, 10⁻⁸ M dexamethasone is employed to robustly activate the GR in cells in vitro. The GR is expressed in B-13 cells, is transcriptionally functional and its activity and apparent ability to drive B-13/H formation at 10nM dexamethasone is blocked by GR antagonists.^{90,119} In primary pancreatic rodent and human acinar cell cultures, significantly higher (10⁻⁶ to 10⁻⁵ M) concentrations are required (and produce a quantitatively much reduced trans-differentiation to hepatocyte-like phenotype).^{87,90} The reason(s) for this are unclear. Although there is another receptor protein for dexamethasone in the rat with a lower affinity for dexamethasone (K_D = 59nM¹¹⁸), the antagonist RU486 does not block dexamethasone binding to this protein, whereas RU486 blocks primary pancreatic rodent and human acinar cell trans-differentiation to hepatocyte-like cells.^{23,92} These observations therefore suggest that the activated GR may be hyper-functional in B-13 cells, although the reason(s) for this remain to be elucidated.

Exposure of B-13 cells to glucocorticoid converts the majority of the cells into a non-replicative B-13/H phenotype, with often less than 5% of the cells remaining in a B-13 phenotype. From a practical point of view, a striking feature of hepatocyte-like cell generation from B-13 cells is the near complete trans-differentiation of B-13 cells to B-13/H cells in response to glucocorticoid. It is therefore possible to generate a near pure population of hepatocyte-like cells with relative ease. This contrasts with the potential for ESCs and iPSCs to differentiate into a variety of cells, although protocols are beginning to address this problem and hepatocyte purity has increased from early reports.

Although the liver has the capacity to regenerate in vivo through mitosis of existing hepatocytes in vivo, hepatocytes in culture do not readily undergo mitosis in vitro.¹¹⁹ Through additions of growth factors, low seeding densities etc, it is possible to stimulate DNA synthesis in cultured hepatocytes, but hepatocytes rarely complete the cell cycle in vitro and therefore, the cells are not readily expanded in vitro in the way that replicative cell lines may be expanded.^{119,120} In this respect, it is possible that the de-differentiation of hepatocytes when they are cultured under conditions designed to promote

growth, has the effect of pushing them into the cell cycle from which they cannot emerge.¹²¹ In contrast, avoiding this regenerative-like response through culturing cells in sandwich cultures of extracellular matrix and/or 3D matrices may explain their superiority in maintaining hepatic function in culture [51-53].¹²²⁻¹²⁴

B-13 cells are resistant to differentiation into other cell types in response to typical differentiating factors such as soluble growth factors and cytokines. In the liver, the progenitor cell (termed oval cell in rodents) is bi-potential and capable of differentiating into either hepatocytes or biliary epithelial cells.^{85,86} It is thought that oval cells only give rise to significant numbers of hepatocytes in liver regeneration when hepatocytes are prevented from undergoing mitosis themselves.^{85,86} If the B-13 cell is derived or markedly related in function to a pancreatic/hepatic progenitor, then one might expect that the B-13 cell be capable of differentiating into other pancreatic and/or hepatic cell types.

B-13 cells have been directed toward a pancreatic β cell phenotype although there appears to be a requirement for ectopic over-expression of specific transcription factors.^{21,22,125} Treating B-13 cells with glucocorticoid and then EGF also promotes a ductal-like phenotype.⁸⁷

Since it is arguably the case that many cell types can be forced to alter their phenotype into other cell types if the appropriate transcription factors are over-expressed (often referred to as direct lineage conversion¹²⁶:- the induction of pluripotency by c-Myc, Klf4, Oct4 and Sox2^{127,128} being effectively direct linear conversion to pluripotent stem cells / indirect lineage conversion to other mature cells), then it may not be surprising that B-13 cells can be directed into phenotypes other than hepatocytes when given the required non-hormonal signals, if sufficiently potent. However, in all cases so far, the quantitative comparable functional differentiation of B-13 cells into other cell types in response to hormonal factors alone, does not compare with the robust response seen with respect to glucocorticoid and hepatocyte-like cell formation. Thus, B-13 cells resist differentiation into other cell types in response to typical differentiating factors such as soluble growth factors and cytokines.

B-13/H cells are quantitatively similarly functional to freshly isolated rat hepatocytes. As outlined above, a marked feature of B-13/H formation from B-13 cells is their quantitative comparability to primary rat hepatocytes. This feature is relevant in particular, when considering in vitro cell models for studying toxicity mechanisms and potentially using the model to screen for drug and chemical toxicity.

Most chemical toxicities (both cytotoxic and genotoxic) in man are the result of metabolic activation in a target tissue(s) because exposure to directly cytotoxic reactive chemicals are normally avoided.¹²⁹ A major driver of activation pathways is the level of expression of gene products associated with pro-toxin activation. In simplistic terms, expression of genes associated with metabolic activation are required to be expressed at levels at least approximating to those found in the target organ(s) for the cells to be a realistic in vitro model for

toxicity assessment. This remains only an initial requirement since, additional layers of complexity would need to be taken into account (e.g. toxicokinetics, protective pathways etc) before such in vitro models could be used to predict in vivo toxicity.

Conclusions

The B-13 cell line provides an expandable donor-free supply of functional rat hepatocyte-like cells that can be readily used without a requirement for expertise found outside the average toxicology laboratory. There remain some limitations to B-13/H cells, such as the fact that they are rat cells and not human, and that there are limitations in the expression of some transporters such as NTCP. However, given their ease of use and cost effectiveness, on balance B-13 cells have clear value in toxicity testing. Loss of some genes, such as CYP1A2, can be overcome through ectopic expression (e.g. the human gene). Furthermore, understanding the unique features of this cell line, when applied to human stem cell-derived hepatocytes, may also be essential in the drive toward generating more functional and stable hepatocytes in vitro.

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Notes and references

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Abbreviations: AdV, adenovirus; CPSI, carbamyl phosphate synthase; CYP, cytochrome P450; CK-19, cytokeratin 19 (commonly used ductal cell marker); DAPI, 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (a fluorescent DNA stain); DEX, dexamethasone; E2 17 β oestradiol; DMEM, Dulbecco's modified Eagle medium; EMT, epithelial-mesenchymal transition; ENaC, renal sodium channel; ER, oestrogen receptor [rER α , rat oestrogen receptor α ; rER β rat oestrogen receptor β]; GAPDH, glyceraldehyde phosphate dehydrogenase; GFP, green fluorescent protein; GSK, GSK 650394 (an SGK1 inhibitor); ICI, ICI182780 (an ER antagonist); NTCP, Na⁺-taurocholate cotransporting polypeptide; PMP70, 70-kDa peroxisomal membrane protein (ABCD3); PPAR α , peroxisomal proliferator activated receptor α ; RT-PCR, reverse

transcription, polymerase chain reaction; qRT-PCR, quantitative RT-PCR.

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REVIEW

Table 1. Hepatocyte phenotype in B-13/H cells, utility in hepatotoxicity studies – blood proteins, cell structure and metabolism.

Gene	Method of analysis	Comment
<u>Blood proteins</u>		
Albumin	ICC, ²³ RT-PCR, ²⁴ WB, ^{25,27} secretion ELISA ²⁶	Hepatocyte-specific, major blood protein. ³⁰
Transferrin	ICC, ²³ RT-PCR, ²⁴ WB ²⁶	Hepatocyte-enriched, also expressed by other tissues such as the brain and testis. Functions as an iron transporter. ³¹
Transthyretin	ICC, ²³ RT-PCR ²⁴	Hepatocyte-enriched, also expressed by chorioid plexus and retinal pigment epithelium. Functions as a thyroid hormone transporter. ³²
α 1-antitrypsin	ICC ²³	Hepatocyte-enriched, but also expressed in monocytes, macrophages, pulmonary alveolar cells, and by intestinal and corneal epithelium. Protease inhibitor that functions to inhibit elastases as well as other proteases. ³³
A2-macroglobulin (α MG)	ICC, ²⁵ RT-PCR, ²⁵ WB ²⁵	Hepatocyte-enriched, also expressed in macrophages, fibroblasts, and adrenocortical cells. Acute phase response protein, broad spectrin protease inhibitor, functions to inhibit fibrinolysis. ³⁴
Haptoglobin (Hp)	ICC, ²⁵ RT-PCR, ²⁵ WB ²⁵	Hepatocyte-enriched, also expressed in skin, kidney and lung. Acute phase response protein, functions to bind and transport free haemoglobin. ³⁵
α -fetoprotein	ICC ²³	Foetal yolk sac and liver-specific (low levels in intestines), blood protein associated with foetus. ³⁶ Expression in B-13/H cells not confirmed in other studies by RT-PCR. ^{24,29}
Apolipoprotein B	ICC ²⁶	Serves as ligand for the LDL receptor. Full length ApoB100 is expressed specifically by the liver. ³⁷
<u>Cell structure</u>		
Cytokeratin 8 (CK8)	ICC ²³	Structural protein associated with secretory epithelium such as hepatocytes. ³⁸
<u>Metabolism</u>		
Glucose 6 phosphatase	ICC, ²³ RT-PCR ²⁴	Liver enriched, also expressed in, kidney, intestine, and pancreatic islets. ³⁹
Tyrosine aminotransferase	RT-PCR ²⁴	Liver –specific. This gene is transcriptionally regulated by the GR. ⁴⁰
Arginosuccinate synthase	RT-PCR ²⁴	Urea cycle enzyme, expressed predominantly in the liver but is also expressed in most other tissues such as fibroblasts, kidney, heart, brain, muscle, pancreas, and red blood cells. ⁴¹
Carbamyl synthetase (CPSI)	RT-PCR, ²⁴ WB, ^{24,26,27} ICC ²⁷	CPSI is a urea cycle enzyme expressed in rat liver throughout much of the lobule but not in proximal centrilobular hepatocytes. ^{42,43} B-13/H cells positive for CPSI were negative for glutamine synthase by ICC. ²⁸
Glutamine synthase (GS)	ICC ²⁸	Ammonia removing enzyme, GS is expressed in rat liver only in the in proximal centrilobular hepatocytes. ^{42,43} B-13/H cells positive for GS were negative for CPSI. ²⁸
Ornithine transcarboxylase	RT-PCR ²⁵	Urea cycle enzyme. ⁴³
Glucokinase	ICC ²⁸	Expressed in liver and other tissues. ⁴⁴

ICC, immunocytochemistry; RT-PCR, RT-PCR determination; WB, Western blotting; ELISA, enzyme linked immunosorbent assay-based determination.

Table 2. Hepatocyte phenotype in B-13/H cells, utility in hepatotoxicity studies – xenobiotic metabolism and related.

Gene	Method of analysis	Comment
<u>Xenobiotic metabolism and related</u>		
CYP450 reductase	WB ^{45,26}	Essential for functional microsomal cytochrome P450 activity. ⁴⁹ Required for toxic effects of many drugs and chemicals. ¹²⁹
Total CYP450	SPEC ^{45,27}	Major xenobiotic metabolising gene superfamily. ⁵⁰ Reduced CO versus reduced spectra peak at 450nm. It is possible to generate cells with similar levels to rat hepatocytes detectable. ²⁷ Required for toxic effects of many drugs and chemicals. ¹²⁹
Cytochrome b5	SPEC	Unpublished.
Aryl hydrocarbon receptor (AhR)	RT-PCR ⁴⁶	Receptor for polyaromatic hydrocarbon xenobiotics – regulates induction of CYP1 genes. ⁵¹
Aryl hydrocarbon receptor nuclear translocator (AhRNT)	RT-PCR ⁴⁶	Heterodimerizes with AhR, essential for effective AhR-mediated transcriptional function. ⁵¹
CYP1A1	RT-PCR, ⁴⁶ WB, ⁴⁶ FUNC ⁴⁶	Expression induced by AhR activator β -naphthoflavone. ⁴⁶ See also Fig. 6. Required to generate genotoxic products from many polyaromatic hydrocarbons such as benz(a)pyrene. ⁴⁶
CYP1A2	Absent - RT-PCR, ⁴⁶ absent – WB, ⁴⁶ absent – FUNC ⁴⁶	All alleles appear disrupted in cell line. ⁴⁶ Expression of human CYP1A2 mediates PhIP activation to a genotoxic product. ⁴⁶
Constitutive androstane receptor (CAR)	RT-PCR ⁴⁶	Transcriptional regulator of CYP2B induction response. ⁵²
CYP2B1	RT-PCR, ⁴⁶ absent – WB, ⁴⁶ present WB, ²⁶ ICC, ²⁸ absent – FUNC ⁴⁶	CAR activators result in induction in mRNA expression, but translation is lacking. ⁴⁶ See also Fig. 6. Note, CYP2B1 is not constitutively significantly expressed in rat hepatocytes, only after administration of inducers. ⁵³
CYP2B2	Absent - RT-PCR, ⁴⁶ ICC ²⁹	CYP2B2 is constitutively expressed at only low levels in rat hepatocytes. ⁵³
CYP2A1/2	WB ⁴⁵	
CYP2C6	RT-PCR, ^{24,46} WB ⁴⁶	An adult hepatic gene expressed at high and similar levels in both male and female rats. ⁵⁴
CYP2C11	RT-PCR, ^{45,46} WB, ^{45,46} FUNC ⁴⁵	CYP2C11 is the major (male-specific) adult hepatic cytochrome P450. Expression is liver-specific. ⁵⁵ Metabolises methapyrilene to an hepatotoxic product, ¹⁴¹ also observed in B-13/H, but not in B-13 cells. ⁴⁶
CYP2C12	Low - RT-PCR ⁴⁶	CYP2C12 is the major (female-specific) adult hepatic cytochrome P450, Expression is liver-specific. ⁵⁵ Levels of mRNA in B-13/H cells are less than 1% of the levels seen in female rat liver. ⁴⁶
CYP2C13	RT-PCR ⁴⁷	Male-specific isoform, specific to liver. ⁵⁶
CYP2D1	RT-PCR ⁴⁷	
CYP2E1	RT-PCR, ^{24,45,46} WB, ^{24,26,45,48} ICC, ^{26,46} FUNC ⁴⁸	Paracetamol is activated to a toxic metabolite. ⁴⁵
Pregnane X receptor (PXR)	RT-PCR, ⁴⁶ FUNC ⁴⁶	Receptor for drugs and xenobiotics – regulates induction of CYP3A genes. ⁵² Receptor regulates inducible expression of CYP3A sub-family – the orthologous sub-family in humans constitutes the predominant drug metabolising sub-family

		responsible for the metabolism of approx. half of all drugs in man. ⁵⁰
CYP3A1	RT-PCR, ⁴⁶ WB, ^{45,46,26} ICC ²⁹	Inducible isoform. ⁴⁶ See also Fig. 6.
CYP3A2	RT-PCR, ⁴⁶ WB, ⁴⁵ FUNC ⁴⁵	Adult liver-specific isoform, male predominant expression. ⁵⁷
CYP3A9	RT-PCR ⁴⁶	
Peroxisome proliferator activated receptor α (PPAR α)	RT-PCR ⁴⁷	Receptor for peroxisome proliferator drugs such as fibrates – regulates induction of CYP4A genes. ⁵⁸
CYP4A1	Absent - RT-PCR, ⁴⁷ absent – WB ⁴⁷	Many CYP4A genes appear to be disrupted in B-13 cells – no induction of proteins - see Fig. 6.
CYP4A2	Absent - RT-PCR ⁴⁷	
CYP4A3	Absent - RT-PCR ⁴⁷	
Farnesoid X receptor	Low expression	Unpublished. Receptor for bile acids – regulator of bile acid synthesis. ⁵⁹
CYP7A1	WB ²⁶	
Liver X receptor	RT-PCR	Both LXR α and LXR β are expressed in B-13/H cells (unpublished). Transcriptionally functional in B-13/H cells (unpublished). Receptor for oxysterols.
<u>Phase II enzyme systems</u>		
Sulfotransferases (SULTs)		5/14 genes expressed at mRNA level. ⁴⁶ Activates estragole to a genotoxic product in B-13/H cells. ⁷⁹
SULT1A1	ICC, ^{23,29} RT-PCR ⁴⁶	Substrate is 4 nitrophenol. ⁶⁰
SULT1B1	RT-PCR ⁴⁶	Substrate is 4 nitrophenol. ⁶⁰
SULT2B1	RT-PCR ⁴⁶	Substrates are steroids and bile acids. ⁶⁰
SULT4A1	RT-PCR ⁴⁶	
SULT5A1	RT-PCR ⁴⁶	
UDP glucuronyltransferases (UGTs)		5/8 hepatic genes expressed at mRNA level. ⁴⁶
UGT1A1	RT-PCR ⁴⁶	Main conjugating enzyme of bilirubin and drugs such as the anticancer drug irinotecan. Other substrates include estradiol. ⁶¹
UGT1A5	RT-PCR ⁴⁶	Transiently elevated in immature rat liver. ⁶²
UGT1A6	ICC ²³	4 nitrophenol is a substrate. ⁶¹
UGT1A7	n/d	Paracetamol is a substrate. However, expression is low in liver, expressed high in gut. ⁶³
UGT1A8	RT-PCR ⁴⁶	
UGT2B1	RT-PCR ⁴⁶	Substrates include morphine and testosterone. ⁶⁴
UGT2B3	RT-PCR ⁴⁶	

Glutathione transferases
(GSTs)

RT-PCR⁴⁶

15/21 genes expressed at mRNA level..⁴⁶ Catalyze conjugation reactions between reduced glutathione (GSH) and electrophilic substrates.⁶⁵

ICC, immunocytochemistry; RT-PCR, RT-PCR determination; WB, Western blotting; FUNC, functional assay; n/d, not determined; SPEC, spectrophotometric determination..

Table 3. Hepatocyte phenotype in B-13/H cells, utility in hepatotoxicity studies – endobiotic and xenobiotic transporters.

Gene	Method of analysis	Comment
<u>Transporters</u> <u>(endobiotic/xenobiotic related)</u>		
Abcb11 (BSEP)	Low - RT-PCR, ⁴⁶ FUNC, ⁴⁶ ICC ²⁸	Canalicular-localised transporter, mediates bile acid efflux. ⁶⁶ Inhibited by troglitazone. ⁶⁷
Abcb1b (MDR1/pglycoprotein1)	RT-PCR, ⁴⁶ FUNC ⁴⁶	Canalicular-localised transporter, mediates efflux of digoxin, talinolol, quinidine and fexofenadine in man. ⁶⁸ Verapamil is an inhibitor in man. ⁶⁸ Higher mRNA levels in B-13/H than intact rat liver. ⁴⁶
Abcb4 (MDR2/pglycoprotein3)	RT-PCR ⁴⁶	MDR2 is the rodent orthologue of human MDR3. Canalicular-localised transporter that mediates the biliary excretion of phospholipids. ⁶⁹ Similar mRNA levels in B-13/H to intact rat liver. ⁴⁶
Abcc1 (MRP1)	RT-PCR, ⁴⁶ FUNC ⁴⁶	Mediates efflux of leukotrienes, oestrogen conjugates, vincristine, etoposide. ⁷⁰ Normal human hepatocytes lack detectable amounts of MRP1. ⁷¹ Basolateral-localised in normal tissues. ⁷¹ Higher mRNA levels in B-13/H than intact rat liver. ⁴⁶
Abcc2 (MRP2)	Low - RT-PCR, ⁴⁶ FUNC ⁴⁶	Canalicular-localised transporter, mediates efflux of amphiphilic conjugates, such as bilirubin glucuronides, glutathione conjugates, and sulfate conjugates. ⁷²
Abcc3 (MRP3)	RT-PCR, ⁴⁶ FUNC ⁴⁶	Basolateral-localised transporter, ⁷³ mediates efflux of glutathione, sulphate and glucuronide conjugates, such as estradiol-17 β -glucuronide, bilirubin-glucuronides, and etoposide-glucuronide. ⁷³ Higher mRNA levels in B-13/H than intact rat liver. ⁴⁶
Abcc4 (MRP4)	RT-PCR, ⁴⁶ FUNC ⁴⁶	Basolateral-localised transporter, possibly mediates transport of cyclic nucleotides such as cAMP; ⁷⁴ low affinity bile salt export; conjugated steroids and prostanoids. ⁷³ Higher mRNA levels in B-13/H than intact rat liver. ⁴⁶
Abcc5 (MRP5)	RT-PCR, ⁴⁶ FUNC, ⁴⁶	Basolateral-localised transporter, possibly mediates transport of cyclic nucleotides such as cGMP ⁷⁴ although this has been questioned. ^{73,75} Higher mRNA levels in B-13/H than intact rat liver. ⁴⁶
Abcc6 (MRP6)	RT-PCR, ⁴⁶ FUNC ⁴⁶	Basolateral-localised transporter, predominantly expressed in the plasma membrane of the hepatocyte (and to a lesser extent in the kidney). ⁷⁶ Mutations in the gene cause a rare, recessive genetic disease, pseudoxanthoma elasticum. ⁷⁶ Similar mRNA levels in B-13/H to intact rat liver. ⁴⁶
Abcg2 (BCRP)	RT-PCR, ⁴⁶ FUNC ⁴⁶	Canalicular-localised transporter, mediates efflux transport of many chemotherapeutics, and involved in haem, folate and uric acid homeostasis. ⁷⁷ This transporter is often associated with stem/progenitor cells. ⁷⁷ Similar mRNA levels in B-13/H to intact rat liver. ⁴⁶
Slc10a1 (NTCP)	Low - RT-PCR ⁴⁶	Basolateral -localised transporter, mediates uptake of bile salts, sulfated compounds, thyroid hormones, drugs and toxins. ⁷⁸ Protein levels are absent in B-13/H and many other cells in culture, limiting their usefulness in toxicity screening studies. ⁷⁹
Slc22a1 (OCT1)	Low - RT-PCR ⁴⁶	Basolateral -localised transporter, mediates uptake of organic cations. ⁸⁰ Expressed at high levels in liver. ⁸¹
Slc22a2 (OCT2)	RT-PCR ⁴⁶	Basolateral -localised transporter, mediates uptake of organic cations. ⁸¹ Expressed at high levels in kidney. ⁸¹ Similar mRNA levels in B-13/H to intact rat liver. ⁴⁶
Slc22a6 (OAT1)	RT-PCR ⁴⁶	Expressed predominantly in the kidneys and localized exclusively to the basolateral membrane of proximal tubular cells. Mediates the uptake of various small, negatively charged molecules, such as endogenous metabolites, toxicants, and numerous clinically used therapeutics - functions as an antiporter exchanging intracellular α -ketoglutarate against extracellular organic anions and drugs. ⁸² Similar mRNA levels in B-13/H to intact rat liver. ⁴⁶

Slc22a7(OAT2)	Low - RT-PCR ⁴⁶	Expressed in liver and kidney. Apical renal transporter in rat. ⁸² It is unclear why OAT2 in humans and Oat2 in rodents show a basolateral versus apical localization in proximal tubules. The presumed localization of OAT2/Oat2 in the sinusoidal membrane of hepatocytes awaits an experimental proof. ⁸²
Slc22a8 (OAT3)	Low - RT-PCR ⁴⁶	Basolateral renal transporter in rat, also expressed in liver. ⁸²
Slc22a9 (OAT5)	RT-PCR ⁴⁶	Apical renal transporter in rat. ⁸² Similar mRNA levels in B-13/H to intact rat liver. ⁴⁶
Slco1a5 (OATP3)	RT-PCR ⁴⁶	Basolateral-localised transporter, primarily expressed in the choroid plexus in rat. ⁸³ Similar mRNA levels in B-13/H to intact rat liver. ⁴⁶
Slco1a6 (OATP5)	RT-PCR ⁴⁶	Basolateral-localised transporter, primarily expressed in the kidney in rat. ⁸³ Similar mRNA levels in B-13/H to intact rat liver. ⁴⁶
Slco2b1 (MOAT)	Low - RT-PCR ⁴⁶	Transporter for prostaglandins, leukotrienes and taurocholate. ⁸⁴

ICC, immunocytochemistry; RT-PCR, RT-PCR determination; WB, Western blotting.

Table 4. Reports for evidence for hepatocyte formation in the pancreas in vivo.

Condition/treatment	Species	Comments
Pancreatic liver regeneration ⁹³	Hamster	Pancreatic regeneration was induced by maintaining animals on a methionine-deficient and i.p. treatment with 500mg/kg bw/day DL-ethionine for 8 days. On the ninth day, animals were returned to a full amino acid diet and given a single i.p. injection of 800 mg/kg bw methionine. Morphological alterations of cells in pancreas observed.
Pancreatic liver regeneration / methyl clofenapate ⁹⁴	Hamster	Pancreatic regeneration was induced by maintaining animals on a methionine-deficient and i.p. treatment with 500mg/kg bw/day DL-ethionine for 8 days. On the ninth day, animals were returned to a full amino acid diet and given a single i.p. injection of 800 mg/kg bw methionine. Treatment with the peroxisome proliferator methyl clofenapate resulted in peroxisome proliferation and increases in the expression of markers of peroxisome proliferation in the pancreas. Cytochemical localization of catalase and immunofluorescence localization of enoyl-CoA hydratase suggested peroxisome proliferation occurred in pancreatic hepatocyte-like cells and not adjacent acinar, duct and islet cells.
Ciprofibrate ⁹⁵	Rat	Animals were treated with the peroxisome proliferator ciprofibrate at 10mg/kg bw/day for 60-72 weeks. Primarily a morphometric study demonstrating appearance of hepatocyte like cells localized adjacent to islets and penetrating into exocrine tissue.
Copper depletion/repletion ⁹⁶	Rat	Animals were fed a copper-deficient diet containing 0.6% D-penicillamine for 8-10 weeks, followed by copper repletion. Morphometric study combined with immunohistochemical staining for albumin and catalase (in pancreatic hepatocytes) and absence of pancreatic enzymes and hormones. Ciprofibrate also shown to induce peroxisome proliferation in the pancreatic hepatocytes.
Copper depletion/repletion ⁹⁷	Rat	Pancreatic hepatocytes shown to express CPSI and urate oxidase – genes expressed highly in hepatocytes.
Copper depletion/repletion ⁹⁸	Rat	Animals were maintained on a copper-deficient diet containing the copper-chelating agent triethylenetetramine tetrahydrochloride at 0.6% w/w for 7-9 weeks and then returned to normal rat chow. This resulted in low mortality (10%); pancreatic depletion of acinar cells and the development of multiple foci of hepatocytes in all 100% of rats - in some animals, liver cells occupied more than 60% of pancreatic volume within 6-8 weeks of recovery. Albumin mRNA expression confirmed in hepatocyte containing pancreata.
Cadmium chloride ⁹⁹	Rat	Repeated sub cutaneous injections of cadmium chloride resulted in the appearance of pancreatic hepatocytes based on rat albumin and connexin 32 expression.
Dioxin (2,3,7,8-tetrachlorodibenzo-p-dioxin, TCDD) treatment ¹⁰⁰	Hamster	Two or 6 intraperitoneal injections of 100µg/kg bw at 4 week intervals induced pancreatic hepatocytes in 75% and 89% of the animals respectively.
Copper depletion/repletion ¹⁰¹	Rat	Pancreatic hepatocytes appear in both male and female pancreata in this model. Male pancreatic hepatocytes synthesize in all cells, the male liver (centrilobular-specific) alpha 2u-globulin under androgenic regulation. Expression was reduced after orchietomy.
Copper depletion/repletion ¹⁰²	Rat	CPSI and glutamine synthetase are co-expressed in pancreatic hepatocytes, in contrast to the liver where they show marked demarcation across the hepatic lobule. Expression confirmed at mRNA level
Copper depletion/repletion ¹⁰³	Rats	Carcinogens that cause nucleolar segregation in parenchymal cells of liver, also have the same effect in pancreatic hepatocytes. Dietary administration of 2-acetylaminofluorene (0.025%) for 12 to 32 weeks also led to the development of glutathione S-transferase-P-positive pancreatic hepatocytes in the pancreas. Oval cell proliferation was observed in close association with pancreatic hepatocytes.
Copper depletion/repletion ¹⁰⁴	Rats	Expression of liver-enriched transcription factors during oval cell proliferation in the pancreas preceded the expression of albumin mRNA and subsequent differentiation of hepatocyte phenotype
Genetic model - ectopic expression of keratinocyte growth factor ¹⁰⁵	Mouse	Insulin promoter-regulated KGF resulted in the appearance of hepatocytes within the islets in the pancreas.

Genetic model – ectopic expression of FGF8 ¹⁰⁶	Mouse	Glucagon promoter-regulated expression of FGF8 resulted in the appearance of hepatocyte-like cells in the periphery of pancreatic islets.
Dexamethasone treatment ²⁴	Rat	Administration of dexamethasone i.p. resulted in the appearance of occasional hepatocytes in the pancreas after 3 weeks.
Genetic model of elevated systemic glucocorticoid levels (Cushing's disease) ¹⁰⁷	Mouse	Pancreatic hepatocytes appear by 21 weeks of age as determined by transcript analysis, WB and ICC. Adrenalectomy prevents appearance of pancreatic hepatocytes.
Genetic model – ablation of Sox9 ¹⁰⁸	Mouse	Pancreatic progenitor-specific ablation of Sox9 during early pancreas development caused pancreas-to-liver cell fate conversion
Resected human pancreas from patients maintained on long term systemic glucocorticoid therapy ⁹⁰	Human	Pancreatic hepatocytes observed in resected human pancreas from patients maintained on long term systemic glucocorticoid therapy, as determined by transcript analysis, WB and ICC.

Note that the isolation and culture of rat or mouse acinar cells in vitro and treatment with glucocorticoid is also capable of inducing the expression of genes associated with the hepatocyte,^{138,139} although the levels of expression are not comparable to those seen in B-13/H cells.

Figure 1. Origin of the AR42J and AR42J-B13 (B-13) cell lines. Upper panels, light micrographs of the indicated cell line, lower panel, fluorescence immunocytochemical staining as outlined,⁴⁸ note cytoplasmic staining of the CYP2E1, DAPI staining DNA and identifies the nucleus.

Figure 2. Localisation of putative synaptophysin positive progenitor cells in rat liver and pancreas. **A**, schematic diagram of pancreas and liver tissue and potential analogous position of putative progenitors in the respective tissues. **B**, immunohistochemical staining for synaptophysin and DAB detection in rat pancreas (left panels) and liver (right panels) stained essentially as outlined.¹³⁰ Dotted panel is expanded in lower panel; d, duct, pv, perivenous region; p, putative progenitor.

Figure 3. Isolation and culture of putative progenitor cells from rat liver results in generation of duct cells and fibroblasts. Putative progenitor cells were isolated from pronase digestion of the biliary tree remaining after a two-step collagenase digestion of rat liver¹³¹ **A**, Light micrographs (upper panels) of cell types observed after 3 days of culture. Lower panels, immunocytochemical staining for progenitor (OV-6), ductal (CK-19 and fibroblast (vimentin) markers¹²⁹ in the morphologically distinct cell types after 3 days of culture, stained essentially as described.⁴⁸ **B**, RT-PCR analysis for the indicated transcript essentially as described.^{24,48}

Figure 4. Oestrogens do not affect B-13 cell differentiation via the ER or GR. Recent work has demonstrated that B-13 cells are capable of differentiating into ductal-like cells⁸⁷ and that an alternative nuclear receptor – the oestrogen receptor- α (ER α) – mediates the effects of glucocorticoids in lumen-forming cells (such as the progenitor cells of the liver, which are bi-potential and capable of forming hepatocytes or the lumen-forming cholangiocytes).¹³² **A**, demonstrates that the mRNA for ER α as well as the related ER gene ER β are not detectable in B-13 cells in contrast to rat hepatocytes (which are known to express ER α ¹³³) and uterus and the ovary (which has previously been reported to express the ER β ¹³⁴). RT-PCR analysis for the expression of ER α and ER β mRNA transcripts in 4 separate B-13 cell cultures, after 30 cycles essentially as described.^{24,48} **B**, transfection of an oestrogen receptor-responsive reporter gene construct (ERE₃-pGL3promoter)¹³⁵ into B-13 cells and treatment with either DEX or the natural endogenous oestrogen receptor activating ligand 17 β oestradiol (E2) resulted in significant ERE₃-pGL3promoter reporter gene expression in response to DEX*. However, only the GR antagonist RU486 - and not two oestrogen receptor antagonists (tamoxifen or ICI182780) significantly inhibited DEX-dependent increases in ERE₃-pGL3promoter reporter gene expression⁸. These data therefore suggest that DEX-dependent activation of the GR is responsible for the increase in ERE₃-pGL3promoter reporter gene expression, likely due to high GR expression in B-13 cells. This is supported by alterations in phenotype (**C**) or expression of hepatic markers (**D**) as determined by Western blotting.^{24,48}

Figure 5. Over-expression of SGK1F induces a trans-differentiation of B-13 cells to B-13/H cells without the addition of glucocorticoid. B-13 cells were infected with an adenovirus encoding the human SGK1F isoform (AdV-SGK1F) which has been previously shown to direct B-13 differentiation into B-13/H cell,⁹² or a control adenovirus encoding NTCP (AdV-control) and morphology examined after 3 days (**A**) or cells harvested at day 5 for Western blotting (**B**) for the indicated protein.^{24,48} Where indicated, cells were also treated with dexamethasone (DEX), the GR antagonist RU486 or the SGK1 antagonist GSK 650394 (GSK).¹³⁶ For more details on variant SGK1 isoforms and methodology, see Wallace et al, including supplementary data.⁹² **C**, Schematic diagram illustrating the cross talk between the GR, SGK1 and WNT signalling pathways operating in B-13 cells.

Figure 6. Induction of hepatic cytochrome P450s in B-13, B-13/H and rat hepatocytes in vitro. B-13, B-13/H and rat hepatocytes in culture were treated with established cytochrome P450 inducers daily for 3 days (androsthenol, 5 μ M; β -naphthoflavone [β -NF], 20 μ M; phenobarbitone [PB], 1mM; 1,4-Bis-[2-(3,5-dichloropyridyloxy)]benzene, 3,3',5,5'-Tetrachloro-1,4-bis(pyridyloxy)benzene [TCPOBOP], 1.5 μ M; dexamethasone [DEX], 10 μ M; pregnenolone 16 α carbonitrile [PCN], 2 μ M or bezafibrate, 250 μ M as outlined,⁴⁶ followed by Western blotting for the indicated protein.^{24,48}

Figure 7. PPAR α -dependent peroxisomal proliferation, but not CYP4A induction, is functional in B-13/H cells. DNA from B-13 cells was subjected to mate-pair library construction and SOLiD Whole Genome sequencing and analysed as outlined,⁴⁶ **A**, illustrates significant insertions/deletions in the CYP4A locus which

likely explains lack of constitutive and bezafibrate-inducible expression of CYP4A genes as determined by RT-PCR (**B**), qRT-PCR for CYP4A1 mRNA; primary hepatocytes and B-13/H cells were treated for 3 days daily or over 14 days (B-13/H cells only) with vehicle (0.1% (v/v) DMSO) or 250µM bezafibrate in DMSO from a 1000-fold molar concentrated stock (**C**), bars are the mean and standard deviation of 3 independent determinations from a single experiment, typical of at least 3 separate experiments, *indicates significant difference compared to the indicated group at $p < 0.05$. and Western blotting (**D**), cells were treated for 3 days as outlined for B and harvested 24 hours after the last treatment, results typical of 3 separate experiments. All methodologies have been previously published.^{24,46,48} **E**, peroxisomal proliferation in response to bezafibrate treatment in B-13/H cells. B-13/H cells were treated with vehicle (0.1% (v/v) DMSO) or 250µM bezafibrate for 14 days. Following treatment cells were immunostained for the peroxisomal membrane protein 70 (PMP70) marker¹³⁷ essentially as previously described.⁴⁶ The cells were then counterstained with DAPI. Cells were photographed using a Zeiss fluorescence microscope and staining quantified using ImageJ particle counter. Results were normalised to cell number. Results are the mean and SD of 5 fields of view from the same experiment, typical of at least 3 separate experiments. Scale bar indicates 48µm. *significantly different to vehicle control at $p < 0.05$.

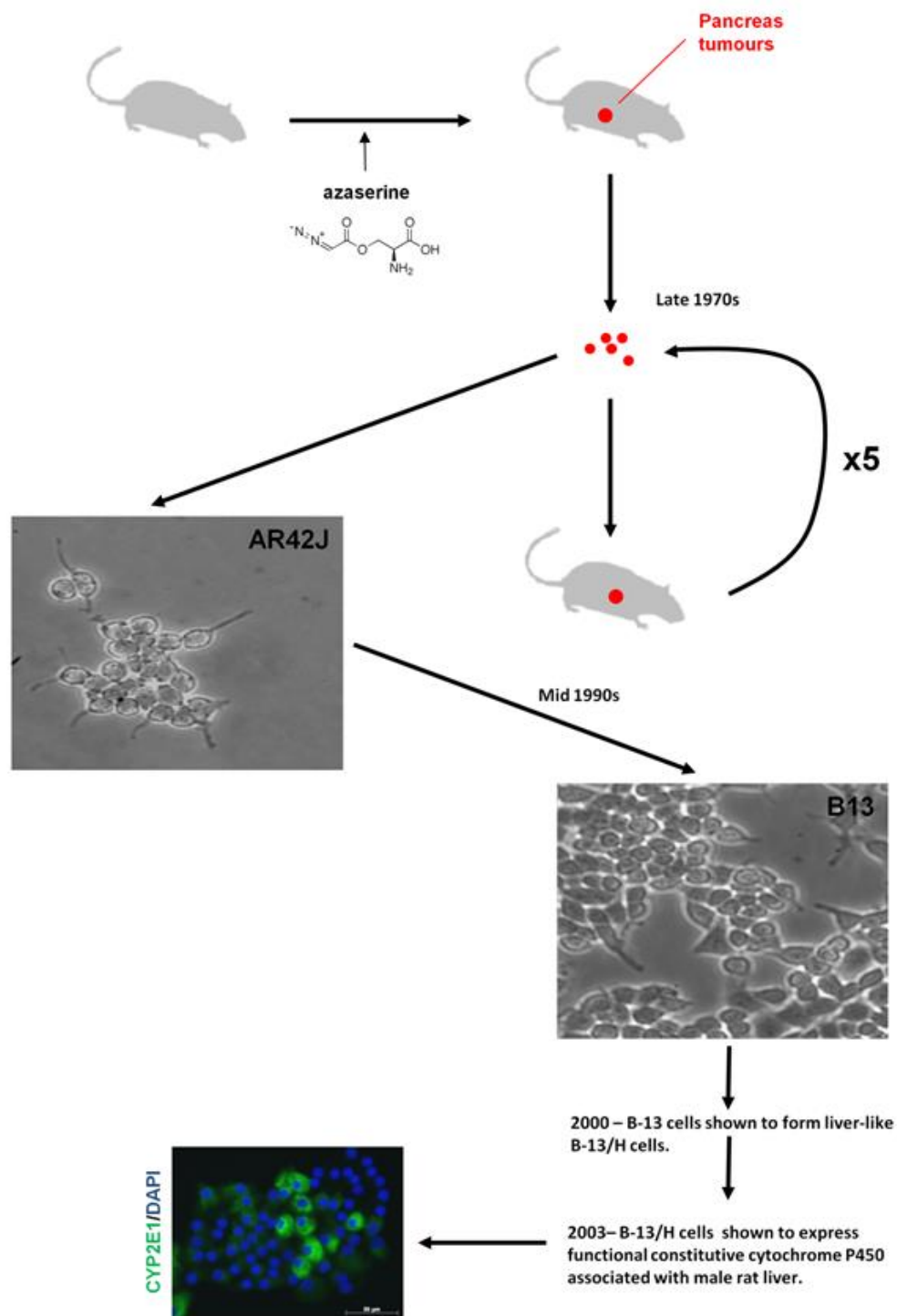
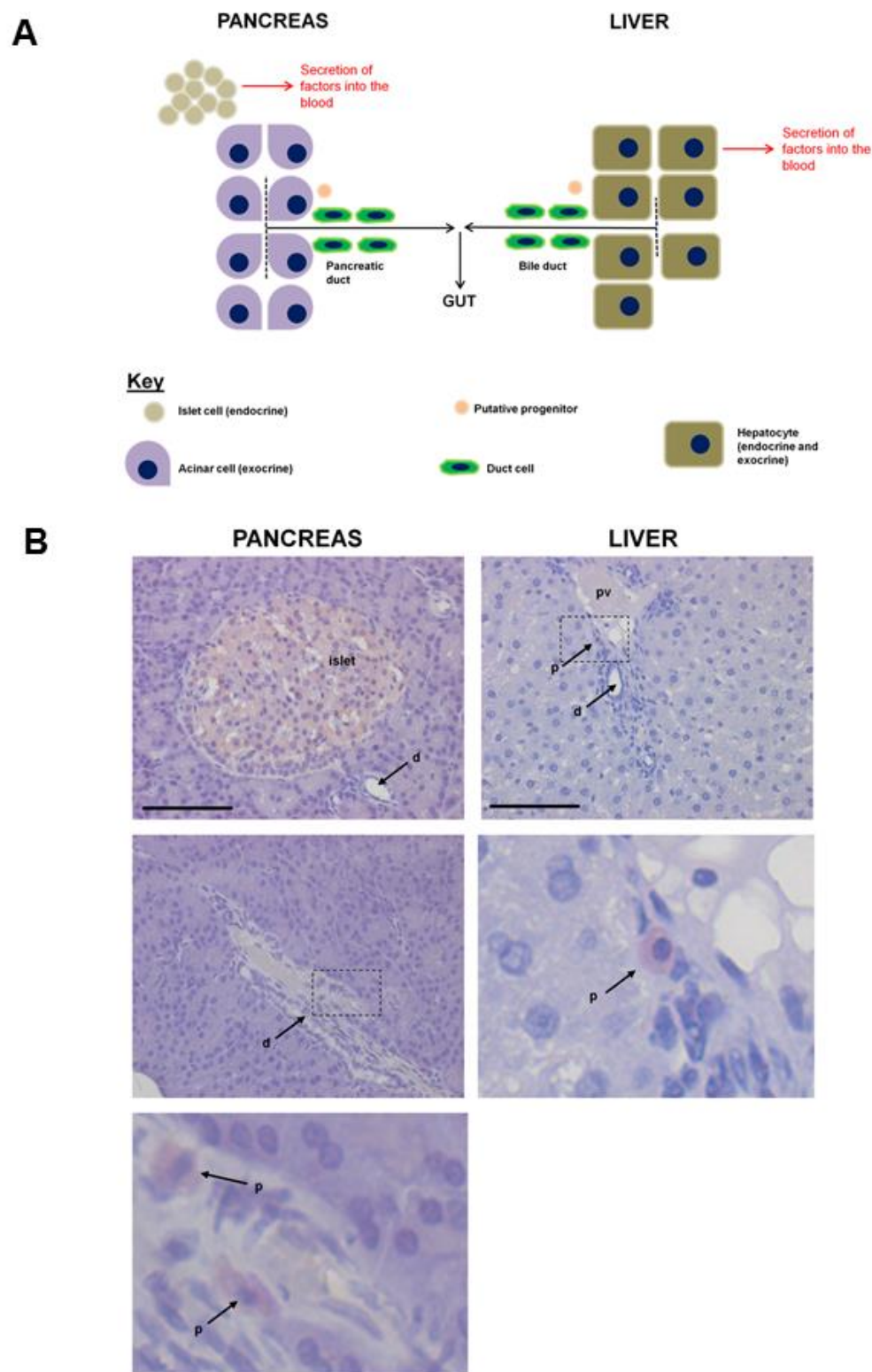


Figure 1

**Figure 2**

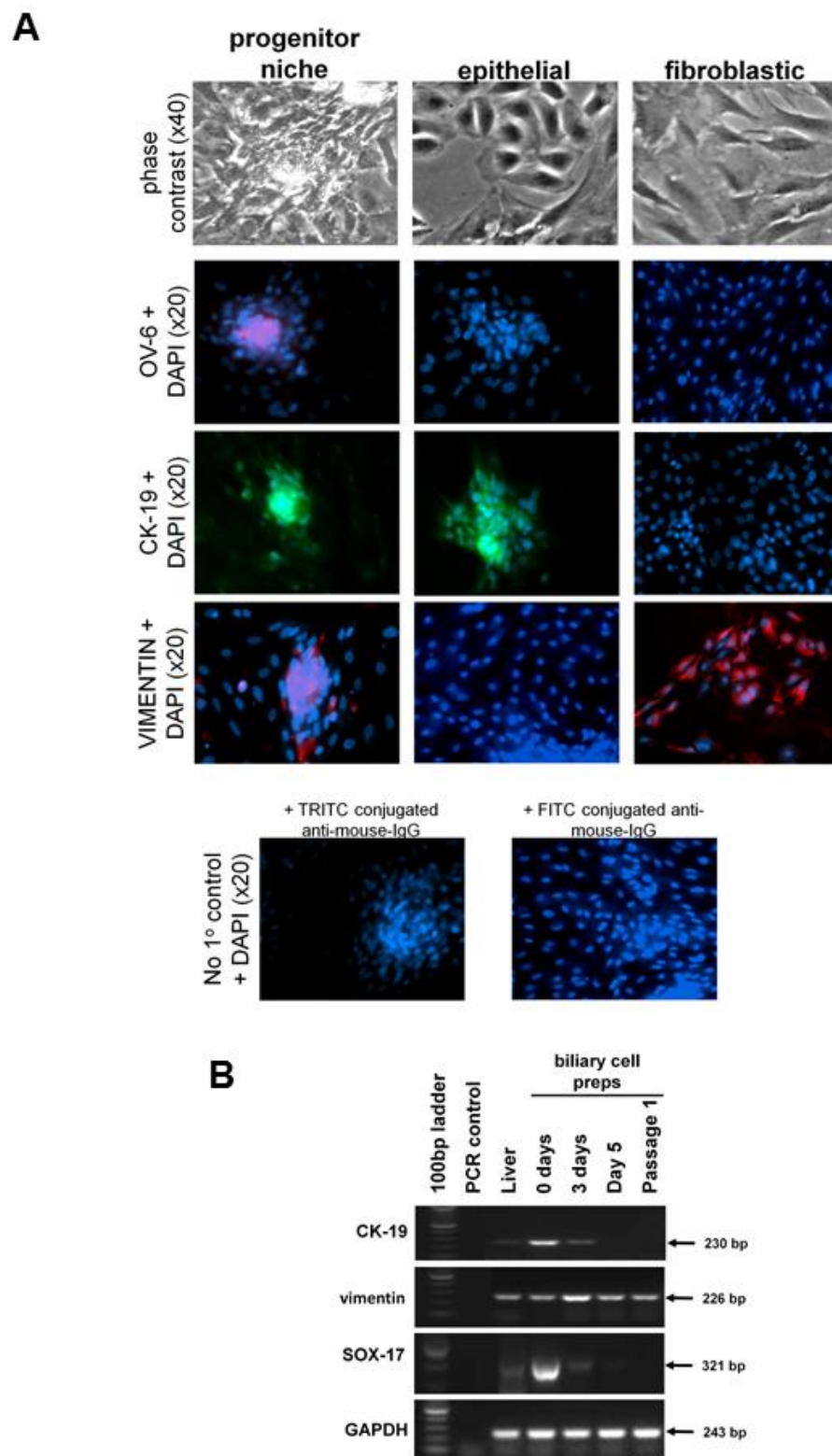


Figure 3

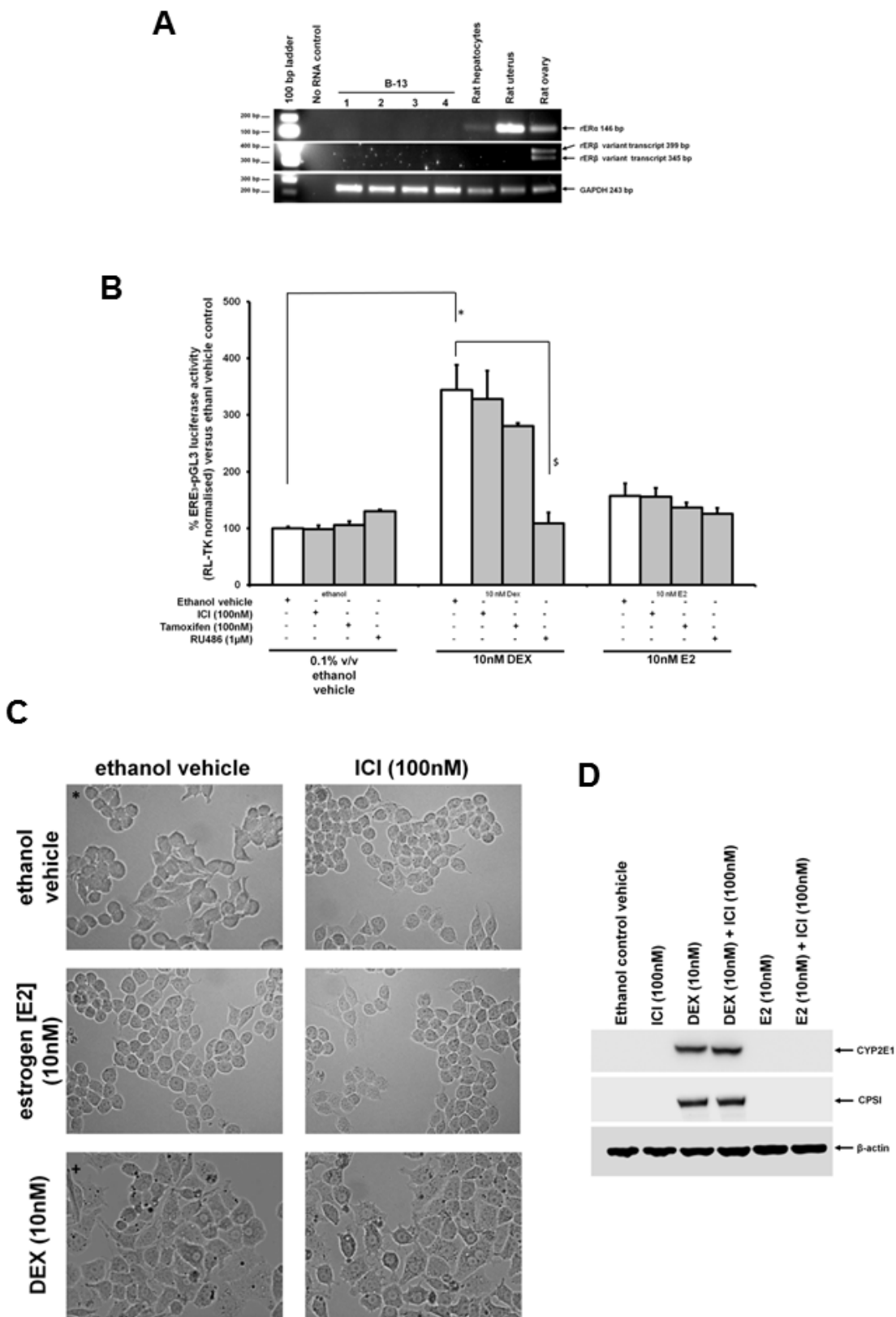


Figure 4

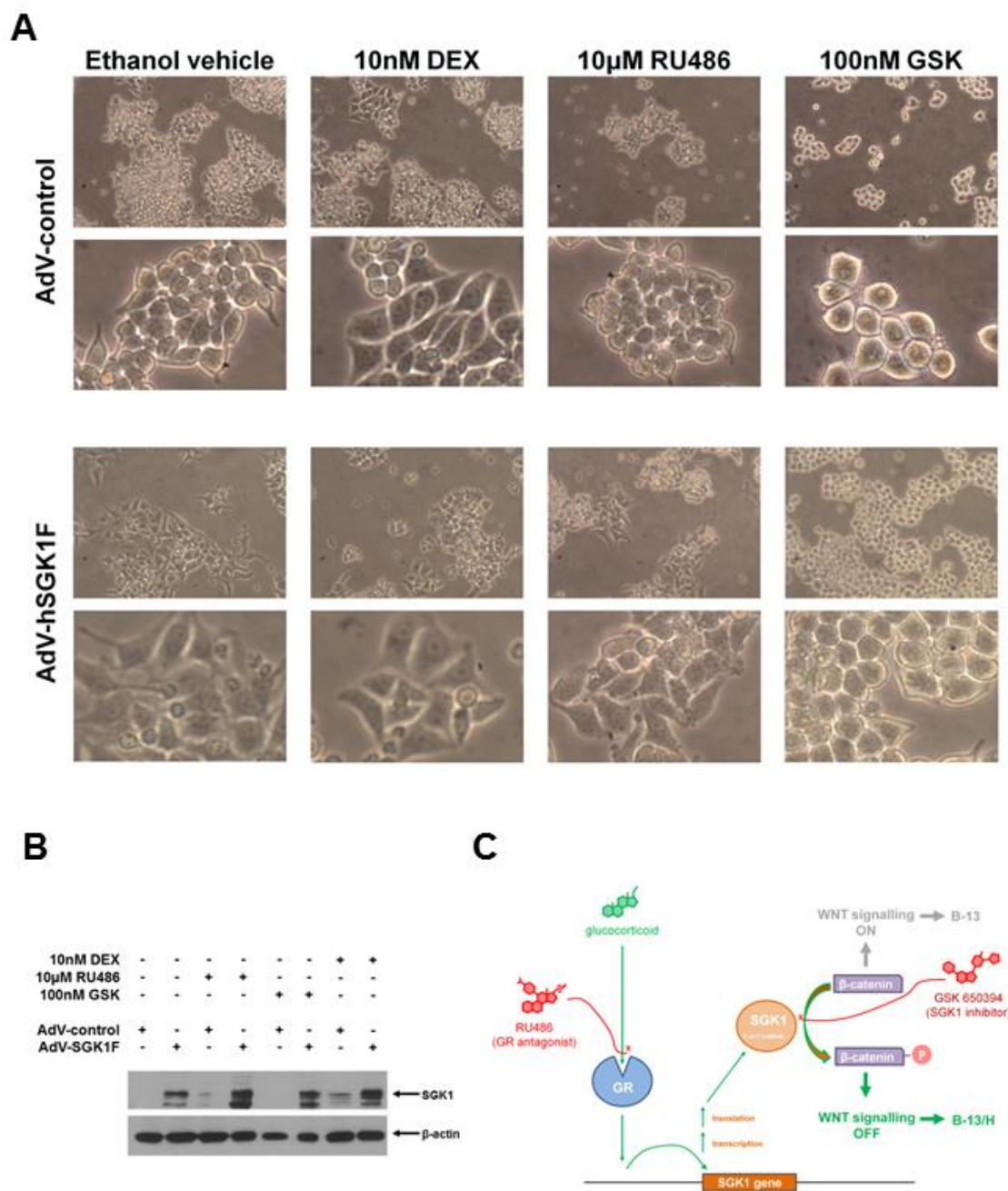
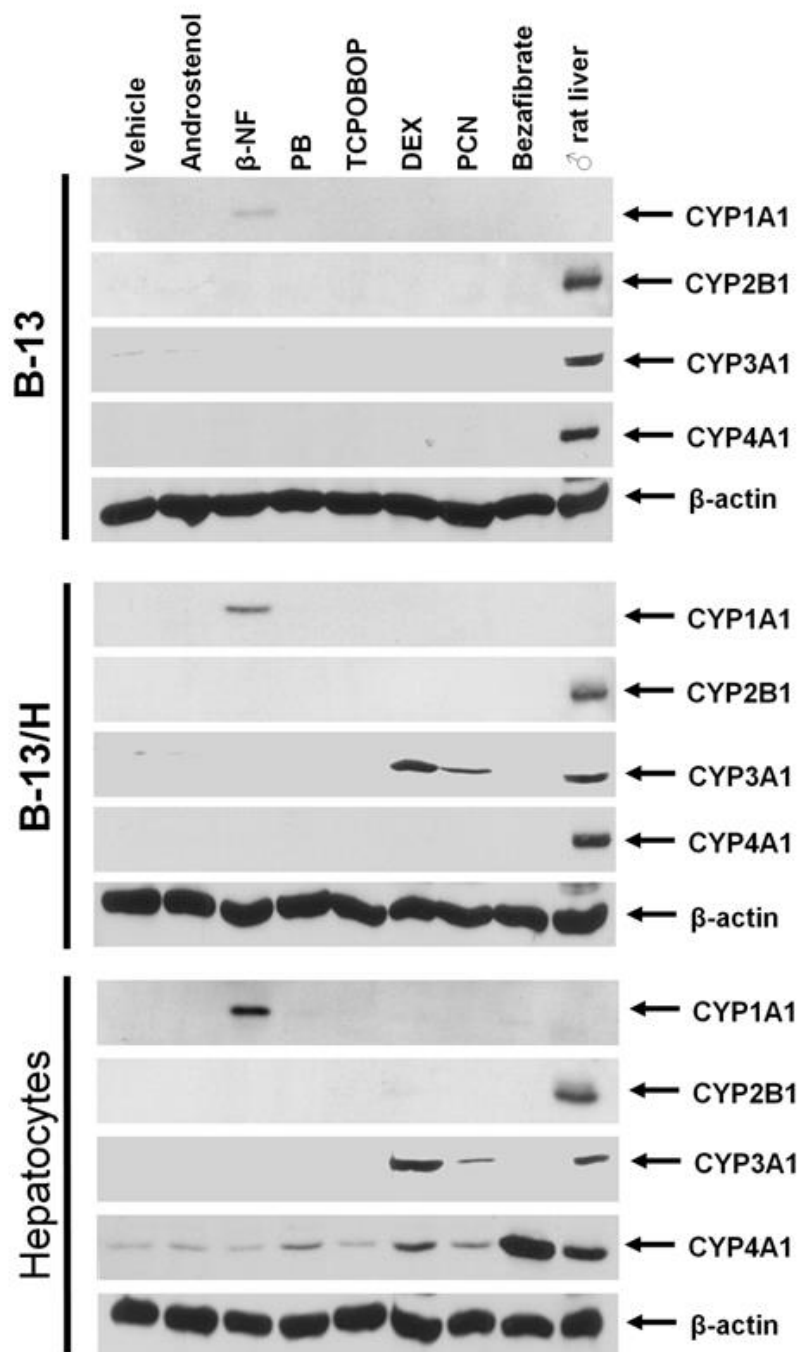


Figure 5

**Figure 6**

